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Interleukin-1 receptor antagonist halts the progression of established crescentic glomerulonephritis in the rat

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Interleukin-1 receptor antagonist halts the progression of established crescentic glomerulonephritis in the rat. The pathogenic role of interleukin-1 (IL-1) in the progression of established rat crescentic glomerulonephritis was investigated by administration of the interleukin-1 receptor antagonist (IL-1ra). Passive accelerated antiglomerular basement membrane (GBM) disease was induced in three groups of six rats. One group was killed on day 7 with no treatment. The other groups received a constant infusion of IL-1ra or saline from day 7 until being killed on day 21. All animals developed moderate glomerular injury, a significant loss of renal function and marked histological damage including crescent formation by day 7. Saline treated animals showed a significant deterioration in these parameters over days 7 to 21. In contrast, animals treated with the IL-1ra over this period showed stabilization of glomerular injury (proteinuria; $P < 0.001$) and a recovery of normal renal function (creatinine clearance; $P < 0.05$). Histologically, IL-1ra treatment suppressed glomerular cell proliferation (PCNA expression; $P < 0.001$) and significantly inhibited crescent formation ($P < 0.005$), glomerular sclerosis ($P < 0.005$), tubular atrophy ($P < 0.05$) and interstitial fibrosis ($P < 0.05$). A key finding was that IL-1ra treatment not only stopped renal leukocyte accumulation over days 7 to 21 ($P < 0.01$), but that treatment also suppressed immune activation of the infiltrate ($P < 0.01$). In conclusion, this study provides direct evidence that IL-1 plays a key role in the progressive/chronic phase of renal injury in experimental crescentic glomerulonephritis and indicates that IL-1ra treatment may be of therapeutic benefit in human rapidly progressive crescentic glomerulonephritis.

Interleukin-1 (IL-1) is a cytokine which elicits a wide range of pro-inflammatory and immunologic effects, including: activation of endothelium; stimulation of T and B cell activation; up-regulation of leukocyte adhesion molecule expression by many cell types; and the induction of a range of cytokines and growth factors including interleukins 1, 2, 6 and 8, tumor necrosis factor-α, monocyte chemotactic protein-1, platelet-derived growth factor, and transforming growth factor-β, which regulate events such as leukocyte chemotaxis and the fibrotic response [1, 2]. A potential role for IL-1 in proliferative forms of glomerulonephritis was first suggested by studies in which macrophage-derived IL-1 was found to stimulate mesangial cell proliferation *in vitro* [3]. Since then, renal IL-1 production has been detected during acute and progressive/chronic phases of experimental [4–10] and human glomerulonephritis [11–13]. The main source of renal IL-1 production appears to be infiltrating macrophages [5, 7, 10–13] which are a common feature of almost all forms of human and experimental glomerulonephritis [14], although other renal cell types such as mesangial cells and tubular epithelial cells can also synthesize IL-1 [12, 15].

To demonstrate a pathogenic role for IL-1 in glomerulonephritis it is necessary to block the action of IL-1 *in vivo*. Such an approach has been made possible by the identification and characterization of a specific IL-1 receptor antagonist (IL-1ra) [1, 16, 17]. Initial studies using the IL-1ra targeted acute glomerular injury in rat anti-GBM glomerulonephritis which is mediated by a transient glomerular neutrophil influx following deposition of antibodies on the GBM [18–20]. IL-1ra treatment during this period had no discernible effect upon neutrophil influx or glomerular injury. However, an important finding was that a 14 day treatment with the IL-1ra from the time of anti-GBM serum injection produced a marked suppression of the monocyte-dependent phase of glomerular injury and renal impairment [20]. Having demonstrated a key role for IL-1 in the induction of monocyte-dependent renal injury, the next question was whether blocking IL-1 could intervene in the progressive phase of established crescentic glomerulonephritis. This is an important issue because of its relevance to treatment of human disease and in identifying pathogenic mechanisms of renal damage, as a range of other mediators of renal injury are produced during the progressive phase. Hence, this study examined the ability of IL-1ra treatment to intervene in the progression of established rat anti-GBM glomerulonephritis.

Methods

Animals

Inbred male Sprague-Dawley rats (150 g) were obtained from the Monash University Animal House.

Nephrotoxic serum

Rabbit anti-rat GBM nephrotoxic serum was raised by repeated immunization of New Zealand white rabbits with particulate rat GBM, as previously described [21]. The anti-GBM serum was pooled, decomplemented and adsorbed extensively against normal rat erythrocytes.

Experimental design

Passive accelerated anti-GBM disease was induced in 18 rats as previously described [20, 22, 23]. Animals were immunized subcutaneously with 5 mg normal rabbit IgG in Freund's complete adjuvant and injected intravenously with 10 ml/kg body weight

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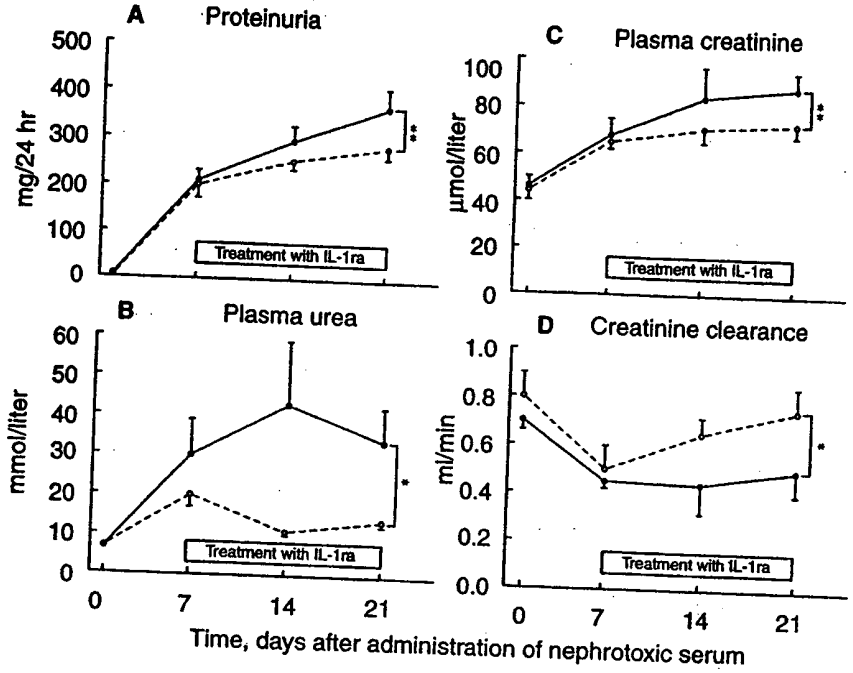

Fig. 1. *Suppression of renal injury by IL-1ra treatment.* Data for (A) 24-hour urinary protein excretion, (B) plasma urea levels, (C) plasma creatinine levels, and (D) creatinine clearance are shown. Symbols are: saline treated animals (●), IL-1ra treated animals (--O--) . Data are expressed as the mean ± SEM for each experimental group of 6 animals. Untreated animals killed on day 7 of anti-GBM disease had proteinuria of 228 ± 25 mg/24 hr, plasma urea of 18.5 ± 1.2 mmol/liter, plasma creatinine of 72.5 ± 5.8 μmol/liter, and creatinine clearance of 0.57 ± 0.08 ml/min. Statistical differences between untreated and IL-1ra treated groups was assessed by one way analysis of variance (ANOVA); * $P < 0.05$, ** $P < 0.001$.

rabbit anti-rat GBM serum (12.5 mg IgG/ml) five days later (termed day 0). One group of six rats was killed on day 7 with no treatment. The remaining animals received a constant infusion of either human recombinant IL-1ra (Synergen, Colorado, USA) or saline from day 7 until being killed at day 21 by means of an Alzet 2002 miniosmotic pump implanted under the skin of the back which delivered 0.5 μl/hr of 50 mg/ml IL-1ra in saline. Following pump implantation, all wounds healed cleanly and there were no signs of infection when pumps were removed at the end of the experiment.

Blood samples and 24-hour urine collections were taken on days 0, 7, 14, and 21. Plasma levels of hrIL-1ra were measured by a commercial ELISA (R&D Systems, MN, USA) and were 671 ± 175 ng/ml (mean ± SEM) on day 14 and 643 ± 112 ng/ml on day 21. In addition, one group of six normal rats was also examined.

Analysis of renal function and proteinuria

Urinary protein excretion was determined using the Manual Ponceau Red method. Urinary blood (hematuria) was determined by a standard Combur's stick test (Boehringer Mannheim) and semi-quantitated into four scores: (1) trace or minor, (2) mild, (3) moderate, and (4) severe. Concentrations of plasma and urine creatinine were measured using the standard Jaffe rate reaction (alkaline picrate), while plasma urea concentrations were measured by the NED/OPA assay. All analyses were performed in the Department of Biochemistry, Monash Medical Centre.

Immunofluorescence

Tissues for direct immunofluorescence staining were frozen in liquid nitrogen and 6 μm cryostat sections were stained with fluorescein isothiocyanate (FITC)-conjugated goat polyclonal antibodies to rat IgG, C3 and fibrinogen or FITC-conjugated sheep anti-rabbit IgG (Nordic, the Netherlands). The intensity of antibody staining was semi-quantitatively assessed as: nil (0), mild (+), moderate (++), and strong (+++). In addition, day 21 tissues were assessed for deposition of immune reactants by semi-quantitative immunofluorescence staining using a blinded antibody titration method as previously described [23]. Briefly, consecutive cryostat sections from each animal were incubated with serial dilutions of FITC-conjugated antibodies against rabbit IgG, rat IgG or rat C3 Blinded sections were examined on the same day and the titer at which antibody staining became undetectably scored. Results are expressed in terms of mean ± SEM of the inverse antibody titer for groups of six animals.

Measurement of plasma antibodies

Plasma levels of rabbit IgG, rat anti-rabbit IgG, rat anti-human rIL-1ra IgG and total rat IgG were determined by capture ELISA as previously described [23]. Briefly, 96-well ELISA plates were coated with 100 μl of swine anti-rabbit IgG, normal rabbit IgG, human recombinant IL-1ra or normal rat IgG (10 μg/ml in 0.1 M carbonate-bicarbonate buffer, pH 9.0) overnight at 4°C, blocked with 2% bovine serum albumin and washed (×4) with 0.05% Tween 20 in PBS. Triplicate serum samples (1:1000 dilution) were added to wells, incubated for two hours and washed (×4) with 0.05% Tween 20 in PBS. Bound rabbit or rat IgG was detected using a peroxidase-conjugated sheep anti-rabbit IgG or peroxidase-conjugated sheep anti-rat IgG (Sigma Chemical Co., St. Louis, MO, USA) and color development with the OPD substrate for 10 minutes in the dark. The reaction was terminated with 3 M H_2SO_4 and optical density (OD) was read at 490 nm on a Dynatec MR 5000 ELISA plate reader. The background reading obtained with normal rat serum, which was the same as the buffer blank, was subtracted from the readings, except for measurement of total rat IgG when the buffer blank was subtracted.

Histopathology

Tissues for histology were fixed in formalin and 4 μm paraffi sections were stained with hematoxylin and eosin or periodic acid-Schiff. Glomerular and tubulointerstitial damage was scored as follows: the percentage of glomeruli exhibiting atrophy/segmental sclerosis, global sclerosis or glomerular crescent formation

was assessed by examination of at least 100 glomerular cross sections per animal in periodic acid-Schiff-stained sections. Glomerular hypercellularity was assessed on the basis of total glomerular cell counts/glomerular cross section (gcs). At least 100 glomeruli per animal were scored in hematoxylin and eosin-stained sections and ranked as follows: (0), normal (less than 50 cells/gcs); (1), mild (60 to 80 cells/gcs); (2), moderate (80 to 120 cells/gcs); (3), severe hypercellularity (more than 120 cells/gcs).

Tubulointerstitial lesions of tubular atrophy and fibrosis were semi-quantitatively analyzed on hematoxylin and eosin-stained sections and graded on a scale of 0 to 3 as follows: (0) no apparent damage; (1) mild damage, with lesions involving less than 15% of the cortex; (2) moderate damage, involving 15 to 30% of the cortex; and (3) severe damage, that is, involving more than 30% of the cortex and focal accumulation of leukocytes at sites of damage.

Immunoperoxidase staining

Monoclonal antibodies (mAb) used for immunoperoxidase staining were as follows: OX-1, leukocyte common antigen [24]; ED1, monocytes, macrophages and some dendritic cells [25]; R73, non-polymorphic $\alpha\beta$ T cell receptor [26]; OX-8, anti-rat CD8, cytotoxic T lymphocytes and NK cells [27]: F17-23-2, MHC class II Ia antigen (RT1-B) [28]; NDS-61, p55 chain of the interleukin-2 receptor (IL-2R) [29]; PC-10, proliferating cell nuclear antigen (PCNA) [30].

Tissues for immunoperoxidase staining were fixed in 2% paraformaldehyde-lysine-periodate and serial 6 μm cryostat sections were labeled with monoclonal antibodies (mAbs) using a standard three layer peroxidase-anti-peroxidase method and developed with diaminobenzidine as previously described [20, 22]. Three layer immunoperoxidase staining with the PC-10 mAb was performed on cryostat tissue sections which were pre-treated with microwave oven heating for 2 × 5 minutes in 0.01 M sodium citrate pH 6.0 at 800 watts as previously described [31, 32]. This treatment facilitates antigen retrieval, thereby increasing the sensitivity of PCNA detection.

Quantitation of leukocytes in tissue sections

Leukocyte subpopulations infiltrating the glomerulus and interstitium were analyzed by mAb labeling of cryostat tissue sections. Cells labeled by each mAb were counted in high power fields (×400) of 20 consecutive glomeruli for each animal (this minimized variation in cell counts caused by differences in glomerular cross section areas). The mean of 20 glomerular counts from each group of six animals was expressed as cells ± standard error of mean (SEM) per glomerular cross section. To assess tubulointerstitial leukocyte infiltration, cortical areas were selected at random. The number of labeled cells was assessed from 20 consecutive high power fields by means of a 0.02 mm^2 graticule fitted in the eyepiece of the microscope. These fields progressed from the outer to inner cortex, avoiding only large vessels, glomerular and immediate periglomerular areas. For each tissue, the same area was examined in serial sections labeled with different mAbs. No adjustment of the cell count was made for tubules or the luminal space. The mean of 20 field counts from each group of six animals was expressed as cells per mm^2 ± SEM.

Scoring of histological changes, immunofluorescence and immunoperoxidase staining was performed on coded slides by an experienced renal pathologist (HYL).

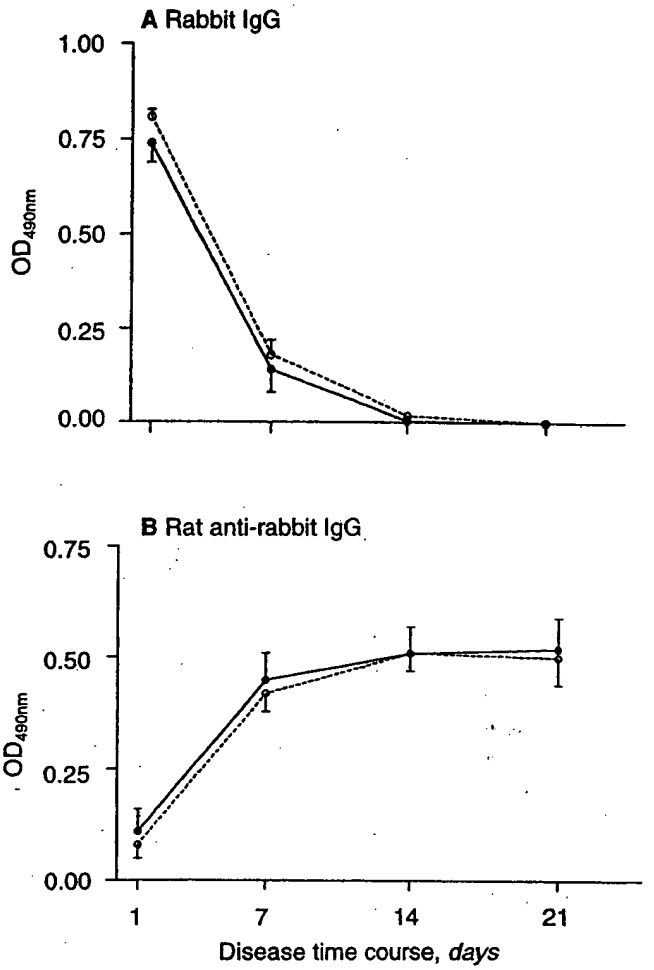

Fig. 2. *Effect of IL-1ra treatment on the plasma antibody levels.* Plasma levels of (A) rabbit IgG, and (B) rat anti-rabbit IgG, were quantitated at different times by ELISA. Symbols are: saline treated animals (-●-); IL-1ra treated animals (--O--). Data are expressed as the mean optical density (OD_{490}) ± SEM for each group of 6 animals with background subtracted. Untreated animals killed on day 7 had 0.85 ± 0.04 and 0.04 ± 0.05 OD_{490} plasma rabbit IgG and 0.05 ± 0.05 and 0.44 ± 0.13 OD_{490} plasma rat anti-rabbit IgG on days 1 and 7, respectively.

Statistical analysis

Data from the measurement of renal function and proteinuria over the experimental course were analyzed using the one way analysis of variance (ANOVA) program from Complete Statistical System (CSS, Statsoft, USA), and individual time points were also compared using the unpaired *t*-test. Data from leukocyte infiltration in the two renal compartments was compared using an unpaired *t*-test. Measurements of hematuria and histological changes were compared by the non-parametric Mann-Whitney U test.

Results

Renal function and proteinuria

On day 7 of anti-GBM disease, all animals exhibited moderate proteinuria, a significant increase in plasma levels of creatinine and urea, and a 37% reduction in creatinine clearance (Fig. 1). Over days 7 to 21 there was a significant deterioration in saline

Table 1. Inhibition of histopathological damage by IL-1ra treatment

	Glomerulus							Tubulointerstitium	
	Hypercellularity %			PCNA$^+$	Sclerosis %		Crescents	Atrophy	Fibrosis
	+	++	+++	*cell/gcs*	Segmental	Global	%	(0–3)	(0–3)
Untreated Day 7	25.3 ± 2.4	24.5 ± 1.6	10.3 ± 1.5	12.8 ± 1.2	21.0 ± 3.8	0.6 ± 0.2	14.0 ± 3.2	1.2 ± 0.2	1.5 ± 0.2
Saline treated Day 21	16.7 ± 3.2[a]	44.5 ± 3.6[b]	29.5 ± 6[a]	12.0 ± 0.9	49.0 ± 5.1[b]	13.8 ± 5.8[b]	58.5 ± 4.9[b]	2.8 ± 0.2[a]	2.7 ± 0.2[a]
IL-1ra treated Day 21	18.2 ± 1.4[a]	25.0 ± 3.5[b]	13.5 ± 2.3	5.8 ± 0.6[bc]	24.5 ± 3.6[a]	3.2 ± 1.1[a]	25.0 ± 4.0[b]	1.3 ± 0.2[b]	1.5 ± 0.2[a]

[a] $P < 0.05$, [b] $P < 0.005$, compared to untreated day 7 animals, [c] $P < 0.001$, compared to saline treated day 21 animals

treated animals which developed severe proteinuria, a further increase in plasma levels of creatinine and urea, while creatinine clearance remained impaired (Fig. 1). This is consistent with previous studies in this model in which a very similar disease progression was evident in untreated animals over this time course [22, 23].

IL-1ra treatment over days 7 to 21 prevented the increase in proteinuria seen in saline treated animals, stabilized plasma creatinine and reduced plasma urea to normal levels (Fig. 1). Of particular note was the finding that IL-1ra treatment produced a gradual recovery to a normal rate of creatinine clearance (Fig. 1D).

On day 7 of anti-GBM disease, all animals exhibited moderate to severe hematuria (2.3 ± 0.8). In saline treated animals, there was an increase in hematuria over days 7 to 21 (3.0 ± 0.4 on day 21). However, IL-1ra treatment resulted in a marked improvement in hematuria with all animals showing only trace to mild hematuria on day 21 (1.0 ± 0; $P < 0.05$ vs. saline treated).

Deposition of immune reactants

Deposition of immune reactants within the kidney was assessed by direct immunofluorescence staining of cryostat tissue sections. In untreated animals killed on day 7 of anti-GBM disease, there was strong (+++) linear deposition of rabbit IgG and moderate (++) linear deposition of rat IgG, and C3 along the GBM. In addition, moderate (++) fibrinogen deposition was seen within Bowman's space in some glomeruli and within the interstitium. At day 21, saline treated animals exhibited strong (+++) linear deposition of rabbit IgG, rat IgG and C3 along the GBM. There was also a strong patchy deposition of fibrinogen, rat IgG and C3 within Bowman's space, crescents and necrotic glomerular capillary tufts and strong fibrinogen deposition throughout the interstitium.

The effect of IL-1ra treatment on the intensity of immune deposits on the GBM on day 21 of anti-GBM disease was assessed by a serial dilution immunofluorescence technique as described in the **Methods** section. There was no difference in the intensity of rabbit IgG deposition on the GBM in saline treated and IL-1ra treated animals (12000 ± 1454 vs. 12800 ± 1905; mean inverse antibody titer ± SEM, respectively). Similarly, there was no difference in the intensity of rat IgG deposition (900 ± 73 vs. 940 ± 39) or C3 deposition (4800 ± 762 vs. 3290 ± 953) in saline and IL-1ra treated animals, respectively. However, IL-1ra treated animals showed little, if any, deposition of rat IgG, C3 and fibrinogen within Bowman's space and had only mild (+) fibrinogen deposition throughout the interstitium.

Quantitation of plasma antibody titers by ELISA was performed to check that all animals received an equivalent dose of rabbit anti-GBM serum and to examine whether IL-1ra treatment had any suppressive effect on the humoral immune response. Figure 2A shows that high plasma levels of rabbit IgG were still evident 24 hours after injection of anti-GBM serum and had almost disappeared by day 7. There was no difference in plasma rabbit IgG levels in any of the experimental groups. The time course of the rat anti-rabbit IgG response is shown in Figure 2B. Rat anti-rabbit IgG was detected on day 1 reflecting the fact that these animals were primed with rabbit IgG. There was a fourfold increase in rat anti-rabbit IgG levels on day 7 and the response remained at this level to day 21. No differences in rat anti-rabbit IgG levels were apparent between the saline and IL-1ra treated groups. Similarly, there was no difference in plasma levels of total rat IgG on day 21 (0.566 ± 0.017 vs. 0.577 ± 0.024 OD_{490} in saline and IL-1ra treated animals respectively; P = NS). Of note was a small antibody response to the administered human IL-1ra on day 21 (0.013 ± 0.004 OD_{490}) which was absent in saline treated animals.

Histopathology

A detailed evaluation of renal histopathology was made on all three groups of animals (Table 1). On day 7 of anti-GBM disease, untreated animals displayed significant renal lesions. These untreated animals exhibited mild to severe hypercellularity in 60% of glomeruli and there was marked glomerular cell proliferation as assessed by PCNA expression (12.8 ± 1.2 vs. 2.0 ± 1.8 PCNA$^+$ cells/gcs in normal rats; $P < 0.01$). Segmental glomerular sclerosis and crescent formation were also evident on day 7 of disease as was mild tubular atrophy and fibrosis (Table 1). In saline treated animals, there was a pronounced deterioration in renal histopathology by day 21 of the disease. Both the percentage and severity of glomerular hypercellularity increased while the number of PCNA$^+$ glomerular cells remained high. In addition, there was a marked increase in glomerular segmental and global sclerosis and crescent formation as well as severe tubular atrophy and fibrosis.

Renal histopathology changes between IL-1ra and saline treatment animals at day 21. IL-1ra treatment essentially halted deterioration of renal histopathology over the days 7 to 21 period (Table 1). The percentage of hypercellular glomeruli and the severity of glomerular hypercellularity was similar in IL-1ra

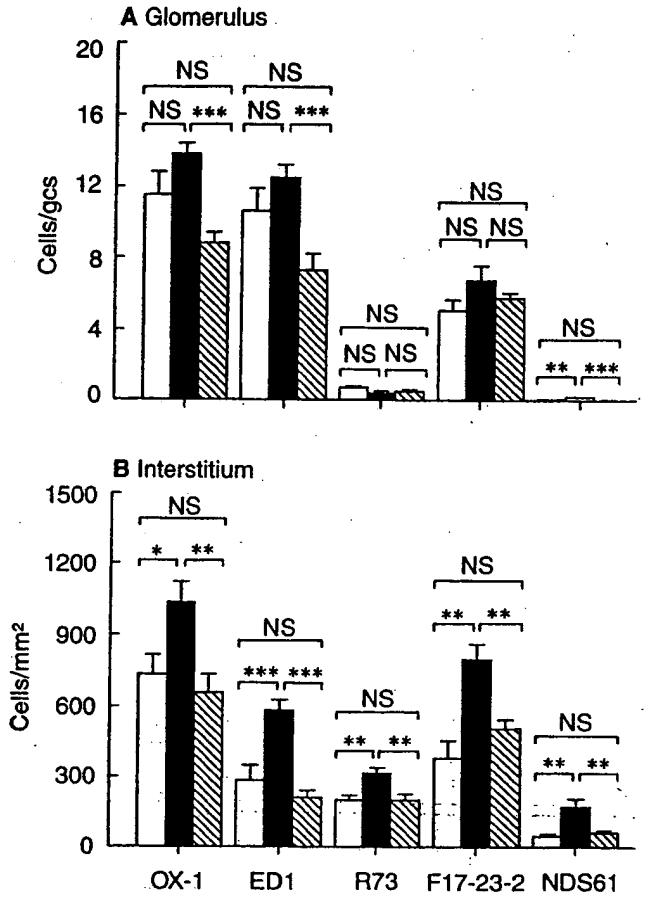

Fig. 3. *Suppression of renal leukocytic infiltration and immune-activation by IL-1ra treatment.* Total leukocytes and leukocyte subsets were analyzed by labeling serial tissue sections with mAbs. Abbreviations are: OX-1, total leukocytes; ED1, macrophages; R73, total T cells; F.17.23.2, MHC class II I-A antigen; NDS 61, IL-2R. (a) Quantitation of leukocyte infiltration in the glomerulus. (b) Quantitation of leukocyte infiltration in the tubulointerstitium. Data are expressed as the mean ± SEM for each group of 6 animals. Open bars represent untreated day 7 anti-GBM disease. Closed bars represent day 21 saline treated animals. Hatched bars represent day 21 IL-1ra treated animals. Data for antibody labeling of normal rat glomeruli are as follows: 0.94 ± 0.10 OX-1$^+$ cells/gcs; 0.76 ± 0.10 ED1$^+$ cells/gcs; 0.34 ± 0.10 R73$^+$ cells/gcs; 1.1 ± 0.21 F.17.23.2$^+$ cells/gcs; 0 ± 0 NDS 61$^+$ cells/gcs. Data for antibody labeling of normal rat interstitium are as follows: 99.4 ± 10.2 OX-1$^+$ cells/mm^2; 26.0 ± 1.4 ED1$^+$ cells/mm^2; 31.4 ± 6.7 R73$^+$ cells/mm^2; 99.0 ± 7.0 F.17.23.2$^+$ cells/mm^2; 0 ± 0 NDS 61$^+$ cells/mm^2. Statistical differences between the different groups was assessed by unpaired *t*-test: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, or not significant (NS).

treated animals at day 21 and untreated animals at day 7, while glomerular cell proliferation was markedly inhibited by IL-1ra treatment (Table 1). Glomerular segmental and global sclerosis and crescent formation were not different in IL-1ra treated animals compared to untreated animals at day 7. Similarly, tubular atrophy and interstitial fibrosis were also unchanged. An example of histological damage in an IL-1ra treated animals is shown in Figure 3b.

Glomerular leukocytic infiltration

Renal leukocytic accumulation and immune activation was examined by mAb labeling of cryostat tissue sections. There was a prominent glomerular leukocyte infiltrate on day 7 of anti-GBM disease (11.4 ± 1.3 vs. 1.1 ± 0.2 OX-1$^+$ cells/gcs in normal rats; $P < 0.001$) which was composed of ED1$^+$ macrophages (Fig. 3a). There was no significant increase in glomeruli R73$^+$ T cells (Fig. 3a) or CD8$^+$ cells (0.53 ± 0.1 vs. 0.24 ± 0.1 CD8$^+$ cells/gcs in normal; P = NS), although there was an increase in the number of CD4$^+$ cells (3.47 ± 0.3 vs. 0.56 ± 0.12 CD4$^+$ cells/gcs in normal; $P < 0.05$) which presumably reflects a subpopulation of CD4$^+$ macrophages. There was also a significant increase in the number of glomerular cells expressing MHC class II antigens (5.1 ± 0.6 vs. 1.2 ± 0.2 MHC class II$^+$ cells/gcs in normal rats; $P < 0.05$) which may be "activated" macrophages or mesangial cells.

In saline treated animals, there was no significant change in the pattern of glomerular leukocytic infiltration or MHC class II expression over days 7 to 21 (Fig. 3a). However, IL-1ra treatment over this period significantly reduced glomerular macrophage infiltration in comparison to saline treated animals (Fig. 3a).

Interstitial leukocytic infiltration

The composition of interstitial leukocytic infiltration was different to that seen in the glomerulus (Fig. 3). On day 7 of anti-GBM disease there was a significant interstitial leukocyte infiltrate (735 ± 81 vs. 102 ± 16 OX-1$^+$ cells/mm^2 in normal rats; $P < 0.001$) which was composed of both ED1$^+$ macrophages and R73$^+$ T cells. There was evidence of immune activation of the mononuclear cell infiltrate as shown by interleukin-2 receptor (IL-2R) expression and the prominent accumulation of MHC class II$^+$ cells within the interstitium (Fig. 3b). The T cell infiltrate contained both CD8$^+$ cells (109 ± 8.5 at day 7 vs. 38.0 ± 8.0 CD8$^+$ cells/gcs in normal; $P < 0.05$) and CD4$^+$ cells (570.5 ± 94 vs. 92.0 ± 23.8 CD4$^+$ cells/gcs in normal; $P < 0.01$), although many macrophages also expressed the CD4 antigen.

There was a significant increase in the number of interstitial ED1$^+$ macrophages and R73$^+$ T cells in saline treated animals over days 7 to 21. Of particular note was the marked increase in the number of immune-activated mononuclear cells—both IL-2R$^+$ cells (↑283%) and MHC class II$^+$ cells (↑111%)—during this period. Many of these activated cells were seen in focal infiltrates of T cells and macrophages around and within damaged tubules and immediately adjacent to areas of Bowman's capsule rupture. However, there was no change in the number of CD8$^+$ cells (109 ± 8.5 at day 7 vs. 103 ± 9.4 CD8$^+$ cells/mm^2 at day 21; P = NS) while the number of CD4$^+$ cells was increased (570.5 ± 94 at day 7 vs. 846.5 ± 41 CD4$^+$ cells/mm^2 at day 21; $P < 0.01$). This indicated that the increased number of R73$^+$ T cells seen in saline treated animals was due to accumulation of CD4$^+$ T cells.

IL-1ra treatment completely stopped interstitial leukocyte accumulation and immune activation over the 7 to 21 day period (Fig. 3b). The suppression of aggressive focal leukocytic infiltrates was associated with a marked reduction in tubular damage and interstitial fibrosis.

Discussion

This study has evaluated the effect of IL-1ra treatment on the progression of established rat accelerated anti-GBM disease—a severe model of glomerulonephritis which exhibits many features similar to that of human rapidly progressive glomerulonephritis. In this model, significant glomerular injury, renal function impairment and histological damage was evident on day 7 which rapidly progressed to a severe form of crescentic glomerulonephritis by

day 21. IL-1ra treatment over days 7 to 21 halted the progression of established disease as demonstrated by: (i) stabilization of proteinuria and reduction of hematuria, (ii) reversal of renal function impairment, and (iii) inhibition of renal histopathological damage. The ability of IL-1ra treatment to intervene and halt disease progression in this model demonstrates a key pathogenic role for IL-1 in crescentic glomerulonephritis. Indeed, the ability to halt disease progression by targeting just one cytokine is most impressive considering that a range of other cytokines and mediators of renal injury are also produced during the disease process [33]. This argues that the cytokine network *in vivo* operates in an interdependent fashion rather than exhibiting functional redundancy as suggested by their many overlapping functions *in vitro*.

The suppression of rat anti-GBM disease by IL-1ra treatment indicates that IL-1 acts at several levels in disease pathogenesis. Possible mechanisms of IL-1ra mediated suppression of disease progression are discussed below.

T cells and macrophages play a crucial role in the induction and progression of experimental anti-GBM disease [22, 34–38]. Glomerular macrophages are associated with glomerular hypercellularity, crescent formation and glomerulosclerosis, while in the interstitium aggressive focal accumulation of activated T cells (IL-2R+) and macrophages is associated with tubular atrophy, Bowman's capsule rupture, granuloma formation and interstitial fibrosis. This disease is different to the NK ($CD8^+$) cell-dependent anti-GBM disease induced in the susceptible WKY rat strain [39], as it features a progressive accumulation and activation of $CD4^+$ T cells within the interstitium, while $CD8^+$ cell numbers do not change during disease progression over days 1 to 21 [22]. The ability of IL-1ra treatment of established anti-GBM disease to reduce glomerular macrophage accumulation compared to saline treated animals and to completely halt interstitial T cell and macrophage accumulation and activation over this period was associated with the stabilization of glomerular injury and the cessation of histological damage. This suppression of leukocyte infiltration and activation is consistent with previous studies in which IL-1ra treatment prevented the development of glomerular, and in particular, interstitial leukocyte infiltration during the induction phase of rat anti-GBM disease by suppressing up-regulation of renal ICAM-1 (CD54) expression [20, 40].

Glomerular hypercellularity is a feature of proliferative forms of glomerulonephritis and results from an increased number of mesangial cells and glomerular leukocyte infiltration [41]. The use of double immunohistochemistry staining has demonstrated that most glomerular $PCNA^+$ cells detected in human and experimental models of glomerulonephritis are proliferating mesangial cells [41, 42]. In our study, IL-1ra treatment appeared to suppress glomerular hypercellularity through inhibition of mesangial cell proliferation as evidenced by the marked reduction in the number of glomerular $PCNA^+$ cells. This could be a direct effect since IL-1 is a mesangial cell growth factor *in vitro* [3]. However, IL-1ra treatment could also act indirectly through suppression of production of other mesangial cell growth factors such as IL-6 or platelet-derived growth factor (PDGF) by mesangial cells or infiltrating macrophages [31, 43].

One further mechanism by which blocking IL-1 action could inhibit rat anti-GBM disease is through modulation of the humoral immune response. In this model, there is a strong systemic immune response to the immunizing rabbit IgG and the subsequent challenge with rabbit nephrotoxic serum [44]. Semi-quantitative immunofluorescence staining found that IL-1ra treatment had no affect upon the deposition of rabbit IgG, rat IgG or C3 on the GBM. In addition, IL-1ra treatment had no measurable effect upon plasma levels of rat anti-rabbit IgG antibody throughout the disease course. Thus, blocking IL-1 did not affect the systemic humoral immune response. Indeed, a mild antibody response to the administered human IL-1ra was detected, consistent with a report that the IL-1ra does not inhibit antigen specific responses *in vivo* [45].

Further studies of established experimental crescentic glomerulonephritis are warranted to determine: (1) whether disease remains suppressed when IL-1ra treatment is stopped, and (2) how rapidly IL-1ra treatment is able to halt disease progression.

In conclusion, this study provides the first direct evidence that IL-1 plays a key pathogenic role in the progressive/chronic phase of renal injury in experimental crescentic glomerulonephritis. This study also demonstrates the therapeutic potential of the IL-1ra for treatment of human rapidly progressive glomerulonephritis.

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